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NEW GENERATION VACCINES

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New and Improved Vaccines Against Meningococcal Disease

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I. BACKGROUND

A. Causative Agent

Meningococcal disease occurs throughout the world in both endemic and epidemic forms. The causative agent is *Neisseria meningitidis*, which is a fastidious, aerobic, gram-negative diplococcus that is able to ferment both glucose and maltose. Its sole natural habitat is the human mucosal membranes, primarily the nasopharnyx, which it usually colonizes without producing any symptoms of disease. Virulent strains that have been isolated from the blood or cerebrospinal fluid are almost always encapsulated, whereas throat isolates may or may not be encapsulated. The capsules are composed of anionic polysaccharides, which are the basis for further subdivision of the species into serogroups. A total of 13 serogroups are currently recognized: A, B, C, D, 29E, H, I, K, L, W135, X, Y, and Z. With the exception of group D, all the capsular polysaccharides (CP) have been chemically and structurally defined [1-5].

Meningococci have been further subdivided into serotypes, subtypes, and immunotypes on the basis of the antigenic specificity of two major outer membrane proteins (OMPs) and the lipopolysaccharide (LPS) [6]. Tsai and Frasch defined five classes of major OMPs based on molecular weight, peptide maps, and electrophoretic behavior [7]. The serotype is based on the class 2 or class 3 OMP (a given strain has one or the other, but not both), and the subtype is based on the class 1 OMP. About 15 to 20 different serotypes, 11 subtypes, and 8 LPS immunotypes have been identified among group B strains. These subcapsular antigens are heterogeneous within a serogroup and are shared across serogroup lines. Although class 5 OMPs and pili are also good antigens, they are extremely variable both in antigenic specificity and in expression and, therefore, are not very useful for classification [8,9]. The suggested nomenclature for specifying the major antigens on a given strain is serogroup:serotype:subtype:LPS type [6]. For example, a serogroup B strain of serotype 15 and subtype 16 and having LPS determinants 3 and 8 is specified as B:15:P1.16:L3,8. The P1 in the subtype designation refers to the class 1 OMP, and 16 is the subtype determinant.

B. Description of the Disease

The clinical manifestations of meningococcal disease are diverse and range from the asymptomatic carrier state to fulminant meningococcemia that can progress very rapidly, often leading to death in 12 to 48 hours from the onset of symptoms. Most systemic

disease, however, is manifest in the form of meningitis, meningococcemia, or both. Meningococcemia may be benign, severe, fulminant, or chronic. Associated with these primary disease states may be a variety of neurological or immunological complications. The clinical aspects of meningococcal disease have been reviewed recently by Gold [10].

In the preantiserum, preantibiotic era, the case/fatality ratio for meningococcal disease was about 65% to 80%. Treatment with antimeningococcal antiserum, introduced in 1908 [11], eventually reduced the mortality to about 20% to 30%, and the level was further reduced to the current level of 4% to 15% by the discovery and use of antibiotics, beginning with the use of sulfanilamide in 1937 [12]. Even with antibiotic treatment, the prognosis for cases of fulminant meningococcemia without meningitidis is quite poor. Case fatality rates, varying from 15% to 71%, have been reported for such cases in recent years [13].

Meningococcal disease primarily affects young children, but the age distribution varies with the serogroup [14] and the serotype of the infecting strain [15]. The peak prevalence of endemic meningococcal disease caused by all serogroups combined is about six months to one year of age, which corresponds to the age when serum antibody levels are lowest [16,17]. During epidemics, the median age of cases increases to the 5- to 20-year-old range [13]. The median age of endemic cases of meningitidis and meningococcemia is generally lowest with group B and highest with groups A and Y [10,17]. Group B serotype 15, sulfonamide-resistant strains, however, tend to cause disease in older children [15].

C. Historical Disease Pattern and Geographic Distribution

Historically, meningococcal disease has occurred worldwide, often in large epidemic waves, with a periodicity of about ten years. These periodic epidemics have been superimposed on a background of endemic disease that is epidemiologically distinct. Endemic disease is usually much more heterogeneous for both the serogroup and serotype of causative strains [13,18]. The attack rate during endemic periods is normally about 1:100,000 to 3:100,000/year in most countries [15,17]. Epidemics, on the other hand, involve attack rates from about 10:100,000 to as high as 400:100,000 to 500:100,000/year and, most often, have been caused by group A strains [13,19,20]. Serogroup B and C strains generally are most prevalent during endemic periods, but they have also been responsbile for epidemics of a reduced scale [21-25]. Epidemic serogroup A disease has largely been absent from the United States and most European countries since 1950, but it is still a major problem in many areas of the world including the meningitis belt in Central Africa and China. Meningococci of serogroups Y and W135 cause a much smaller, but not insignificant amount of disease, and systemic disease caused by serogroups 29E, H, I, K, L, X, and Z is rare.

Currently, over one-half of all meningococcal disease in North America and Europe, including epidemics in Norway and Cuba, is due to group B [26-28]. Epidemics or major outbreaks of meningococcal disease usually involve a single predominant strain or clone [15,29-31]. Certain serotype:subtype combinations have been found to be associated with epidemic group B and group C disease. For group B disease, serotype 2 strains (mostly 2a:P1.2) were most common in the early 1960s [32,33], but were gradually replaced by 2b:P1.2 strains between the late 1960s and about 1980, depending on the country [15,32,34]. More recently, several closely related strains, including types 15:P1.16 [15,30], 4:P1.15 [27,36], and 15:P1.3 [35] have emerged as epidemic strains.

These strains have been shown by Caugant and co-workers [35] to belong to a cluster of genetically very closely related strains, which were named the ET5 complex. Strains of this genetic clone seem to cause epidemics that persist longer than group B epidemics of the past [15]. For group C disease 2a:P1.2 and 2b:P1.2 strains still predominate [34].

D. Deficiencies in Current Vaccines

Currently licensed meningococcal vaccines consist of different combinations of the purified high-molecular-weight CPs from serogroups A, C, Y, and W-135. The group A and group C CP vaccines, which have been very successful, were developed in the late 1960s by Gotschlich and associates [37,38]. These vaccines, in multiple controlled field trials, have provided protective immunity for several years in individuals over the age of 18 months [39]. In an important trial in Finland, the group A vaccine was effective in children as young as six months, but a second dose was required about three months after the first [40]. The A and C vaccines are very well tolerated and have now been given to many millions of people, without any fatalities or serious permanent sequelae. The efficacy of the Y and W-135 CP vaccines has not been proved owing to the low prevalence of disease caused by strains of these serogroups. They were licensed on the basis of molecular size, chemical purity, and their capacity to induce high titers of bactericidal antibodies. Since 1984, a tetravalent A, C, Y, W-135 CP vaccine has been given to all United States military recruits upon entrance into basic training. Since that time there have been no reported cases of Y or W-135 meningococcal disease in that population [32]. This provides some additional evidence for efficacy of the Y and W-135 CP vaccines.

The principal deficiencies of the licensed meningococcal vaccines are the poor immunogenicity of the group C CP in children under 18 months [41,42] and the lack of a group B vaccine. These are important deficiencies because most endemic group C disease occurs in young children, and a high percentage of meningococcal disease in many areas of the world, including North America and Europe, is currently due to group B [15,17]. In addition, development of a group A vaccine that is less expensive and easier to administer would be a significant improvement for use in poor, underdeveloped countries.

E. Applicability of Molecular Approaches

The prospects for resolving the meningococcal vaccine deficiencies using molecular approaches are quite good. The work done with the CP of *Haemophilus influenzae* type b and preliminary work with meningococcal group C CP suggest that the conjugation of the group A and group C CPs to a suitable carrier protein will result in improved immunogenicity in young children.

The solution to the group B problem is less clear. The principal obstacles to a group B vaccine are (a) the poor immunogenicity of the group B CP [43] and (b) the antigenic variation of the principal subcapsular antigens [6]. Experimental group B vaccines that are currently involved in efficacy trials are expected, if successful, to provide only type-specific protection [44,45]. Molecular approaches are expected to facilitate the development of a broadly protective group B vaccine.

Molecular approaches that are being applied to the group B problem include (a) the use of monoclonal antibodies to identify and study those antigens and epitopes that induce protective antibodies; (b) the use of recombinant DNA methods to clone the

structural genes for important protein antigens, to study the antigenic structure of the proteins, and to explore alternative ways of producing and presenting promising antigens or epitopes as vaccines; and (c) the use of modern chemical methods to prepare protein-CP or protein-LPS conjugate vaccines, to study the antigenic structures of the different LPS and protein serotype epitopes, and to chemically modify the group B CP to enchance its immunogenicity. The development of a broadly protective group B vaccine might conceivably be based on a conserved antigen or epitope, or it may involve the use of multiple serotype-specific determinants. In either event, molecular approaches, such as those mentioned earlier, are expected to play an important role.

II. DEVELOPMENT OF A GROUP B VACCINE

A. Group B Polysaccharide Vaccines

Initial efforts to prepare a group B vaccine followed the successful approach used for the group A and group C vaccines. Purified, high-molecular-weight group B CP [a homopolymer of $\alpha(2\rightarrow8)$-linked *N*-acetylneuraminic acid] was prepared and tested at several different doses by Wyle et al. [43], but it was essentially nonimmunogenic in humans. However, it was evident from the serological studies that most normal human sera contain significant levels of anti-B CP antibodies. This suggested that the group B CP could be immunogenic if correctly presented. Crude preparations of B CP, which consist in part of noncovalent complexes with OMPs, had enhanced antigenicity and immunogenicity. These complexes form spontaneously during purification and are presumably held together by hydrophobic bonds formed between hydrophobic regions on the OMP and fatty acids that are linked through diacylglycerol phosphate to the terminus of the CP chains [46]. These observations led to the preparation and testing of noncovalent complexes of B CP and OMPs as a candidate vaccine [47].

Initial tests with such noncovalent complexes in a few volunteers demonstrated the enhanced immunogenicity of both the B CP and the OMPs [47]. The optimal antibody response to the CP was obtained with a 1:3 (w/w) CP/protein ratio [48]. The antibody response to the B CP in vaccines of this type was initially quite encouraging because most individuals responded with an increase in anti-B antibodies (Fig. 1), as measured by a radioactive antigen-binding assay [49]. The antibodies that were induced were bactericidal against group B strains, regardless of serotype [47]. The antibody response, however, was quite transient and limited to antibodies of the IgM class [47,48]. Further studies showed that the antibodies were of low avidity at 37°C [49] and, although bactericidal with rabbit complement, they were not bactericidal with human complement [50]. These properties are shared with the antibodies to B CP that are present at significant levels in normal human serum (see Fig. 1). Why the source of complement is important is unknown, but it suggests that even if a longer-lasting antibody response could be induced, it probably would not result in protective immunity [51].

Attempts by Jennings and Lugowski [52] to improve the immunogenicity of the B CP by covalent linkage to tetanus toxoid were unsuccessful. The antibody response obtained in animals was directed primarily against the linkage point between the CP and the protein [52].

An explanation for the poor antigenicity of the B CP is suggested by the studies by Finne and co-workers [53]. They demonstrated the presence of oligosaccharides with at least eight repeating units of $\alpha(2\rightarrow8)$ linked *N*-acetylneuraminic acid in rat fetal and

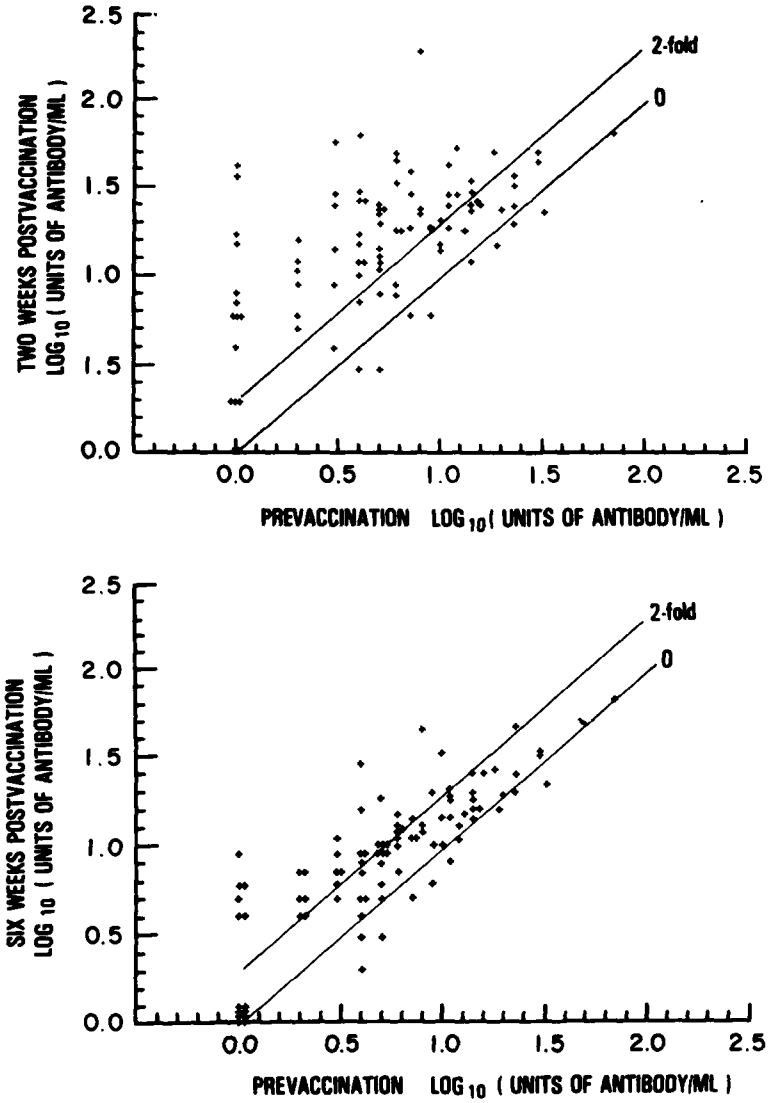

Figure 1 Anti-B polysaccharide antibodies in sera of young adults before and after vaccination with a single dose of vaccine consisting of noncovalent complexes of group B CP and serotype 2a OMPs (lots BP2-WZ-2 and BP2-4). Antibodies were measured by a radioactive antigen-binding assay [49]. Values are based on a standard curve prepared using a reference antiserum that contained 59 μg/ml of anti-B CP antibody by quantitative precipitin assay and was assigned a value of 100 units. Top: comparison of antibody levels at zero and two weeks. Bottom: comparison of antibody levels at zero and six weeks.

newborn brain tissue and, to a lesser extent, in adult brain tissue and newborn kidney, heart, and muscle tissue [54]. Moreover, shorter oligosaccharides of two or three $\alpha(2\rightarrow8)$-linked sialic acid units are known to be present on gangliosides that are found commonly on the surfaces of many animal and human cells.

The immune system does not appear to recognize short, linear oligosaccharides of α(2→8)-linked polysialic acid as foreign, but it does respond to larger conformational epitopes, with transient production of low-avidity IgM antibodies [49,50,55,56]. The implication of this molecular mimicry is that the use of a vaccine based on the B CP might induce autoimmunity. Although this is a legitimate concern, there have been no reports of adverse effects associated with the presence of either natural or vaccine-induced antibody [57,58].

In addition, the B CP is easily degraded by neuraminidase and by weakly acidic pH, which also results in formation of internal esters and a concurrent loss in antigenicity [59,60]. Whether or not rapid degradation of the group B CP in vivo actually occurs and contributes to its poor immunogenicity is unknown.

Detailed studies of the antigenic determinant on the B CP by Jennings et al. [55] and Lifely et al. [56] have shown that the determinant is unusually large for a CP, and it appears to be dependent on conformation. The smallest oligosaccharide capable of efficient inhibition in a primary binding assay was ten residues, and the efficiency of inhibition continued to increase up to 17 residues, and beyond. This is in contrast with oligosaccharides of group C CP which give maximum inhibition at a chain length of five [55].

Jennings et al. [61] have attempted to overcome the intrinsically poor immunogenicity of the B CP by specific chemical modification. The *N*-acetyl groups were removed from the CP by treatment with strong base and replaced by *N*-propionyl groups. This modified structure was subsequently covalently linked to tetanus toxoid. Vaccination of mice with this conjugate resulted in induction of high levels of IgG antibody cross-reactive with the group B CP. The mouse antiserum was bactericidal for all group B strains, independently of serotype. Two populations of antibody were identified in the antiserum. One population reacted with purified group B CP and one did not. The antibodies that did not react with the isolated group B CP appeared to be responsible for the bactericidal activity. Thus, these antibodies were apparently reacting with an epitope on the cell-associated CP that was not present on the isolated CP [62].

Although these results are encouraging and offer the hope of a vaccine effective against all group B strains, there are several important issues yet to be resolved. Addressing these issues will require studies in human volunteers. The first issue is whether or not human beings will respond to the vaccine with IgG antibodies of the same specificities as animals and whether or not these antibodies will be bactericidal with the human complement system [50]. The second issue is that of safety. It appears that the chemically modified CP is able to break the natural tolerance to unmodified B CP. Careful safety studies will be required in the further evaluation of this product.

B. Identification of Important Subcapsular Antigens

Antigens that are able to induce bactericidal antibodies are thought to have a high probability of providing protective immunity. This is because the presence of serum antibodies, and in particular bactericidal antibodies, has been strongly correlated with human immunity [63,64]. In addition to serum bactericidal activity, it is likely that complement-dependent phagocytosis [65] and, possibly, antibody-dependent killing by mononuclear cells [66] play a significant role in the defense against meningococcal disease.

The first attempts to demonstrate that the major OMPs could induce protective antibody in animal models were limited because of a lack of information about the antigenic specificity of the individual OMPs and the LPS. Until monoclonal antibodies became available the "serotype" of a strain was, more or less, a composite of the antigenic specificities of the class 1, class 2 or 3, and class 5 OMPs and, sometimes, the LPS as well [6,67]. Thus, the demonstration by Frasch and Robbins [68] of serotype-specific protection in a guinea pig subcutaneous chamber model and, similarly the results obtained by Craven and Frasch [69] in a mouse bacteremia model and by Ashton and colleagues [70] in a hen embryo model, did not allow one to determine which outer membrane antigen was responsible for the protection. The demonstration of "serotype-specific" protection by subcapsular antigens, however, provided encouragement for further work on the development of these antigens as vaccines.

Monoclonal antibodies to meningococcal subcapsular antigens have been produced in several laboratories [71-79] and have been extremely useful in defining the antigenic specificity, surface exposure, and protective capacity of subcapsular antigens.

The functional activities of monoclonal antibodies to subcapsular antigens have been evaluated in bactericidal assays, phagocytic assays, and animal models of protection. Frasch et al. [77] reported that monoclonal antibodies specific for OMPs of classes 1, 2, and 5 were highly bactericidal. The monoclonal antibodies against the serotype 2 determinant were also found to be protective in a mouse bacteremia model. In a similar mouse model of infection that utilized a mixture of mucin and hemoglobin to enhance the virulence of meningococci for mice, Brodeur and co-workers [76] found that two monoclonal antibodies specific for the serotype 2b determinant on the class 2 OMP were bactericidal and provided strong passive protection. Although the mouse bacteremia models have certain deficiencies, these positive results support the use of the class 2 OMP in group B vaccines.

A somewhat different picture emerged from studies by Saukkonen and associates with an infant rat model [80,81]. A series of different monoclonal antibodies, with specificity for either the class 1 OMP, the class 2/3 OMP, or the LPS, were compared for their bactericidal activity and their capacity to protect infant rats against challenge with a series of different strains of known serotype, subtype, and LPS type. Monoclonal antibodies specific for the group B CP, the class 1 protein, and the LPS showed high levels of protective activity against challenge with homologous strains, whereas those specific for the class 3 serotype protein showed only slight protection. These results were confirmed using pools of seven to nine monoclonal antibodies specific for different class 1 OMPs, class 2/3 OMPs, or LPS. These results suggest that the class 1 OMP and LPS would be effective antigens in a group B vaccine.

Studies of surface exposure of OMPs with the immunogold-labeling technique provide support to the results obtained in the infant rat model. Poolman and co-workers [82] looked at the surface exposure of the major OMPs and LPS under different growth conditions. They found that cells grown to stationary phase produced an additional, larger species of LPS that was not produced by log-phase cells. The class 2 and class 3 serotype proteins appeared to be less accessible to the immunogold label on stationary-phase cells, compared with log-phase cells. The class 1 OMP and LPS determinants, on the other hand, were accessible regardless of growth phase. They concluded that antibody access to the class 2 or 3 OMP may be blocked by the presence of longer LPS chains on the stationary-phase cells.

Table 1 Methods Used for Preparation of Representative Meningococcal OMP Vaccines

Vaccine (Ref)	Vaccine strain (Gp:type:subtype)	OMP source	Detergent used	Method of LPS removal	Residual LPS (%)	Complex with
BP2-WZ-2 (47)	99M(B:2a:P1.2)	Crude B CP + OMC (TES)	CTAB and DOC	Gel filtration	13	B CP
BP2-PA-3 (47)	99M(B:2a:P1.2)	Crude B CP	CTAB	None	ND	B CP
E-06 (83–85)	M986-NCV1 (B:2a:P1.2)	OMC (LiCl)	E-BC	Differential centrifugation	8	None
790309VB (83–85)	M986-NCV (B:2a:P1.2)	OM vesicles (culture SN)	DOC	Differential centrifugation	24	B CP
790626VB (83–86)	M986-NCV (B:2a:P1.2)	OM vesicles (culture SN)	Brij-96 and DOC	Differential centrifugation	16	B CP
B2,7-AC (87)	M986(B:2a:P1.2)	Whole cells (DOC extract)	DOC	Differential centrifugation	ND	A & C CPs

BP2-5-5 (48)	99M(B:2a:P1.2)	OMC (TES)	DOC	Gel filtration	14	B CP
Lot 3179 (44,83,88)	M986(B:2a:P1.2)	OM vesicles (culture SN)	Brij 96 and DOC	Differential centrifugation	10	B CP
ACYW2b15-2 (45,89,90)	44/76(B:15:P1.16) 8047(B:2b:P1.2)	OMC (TES)	Emp. BB	AS precip. in Emp. BB	3	A,C,Y & W135 CP
830207 V (88)	3006(B:2b:P1.2)	OM vesicles (culture SN)	Brij 96	Differential centrifugation	<6	B CP
(a) MP-4 (94) (a) MPC-2	44/76(B:15:P1.16)	Whole cells Z-3,14 extract	Z-3,14	Ion exchange and gel filtration	5	(a) Z-3,14 (b) C CP
MB6-180 (93)	7622(B:6)	Crude B CP	CTAB	Gel filtration	1	B CP

Abbreviations used: CP, capsular polysaccharide; OMC, outer membrane complex; OMV, outer membrane vesicles; TES, tris-EDTA–sodium chloride extraction buffer; SN, supernatant; DOC, deoxycholate; Z-3,14, Zwittergent 3,14; Emp. BB, Empigen BB; CTAB, cetyltrimethyl ammonium bromide; AS precip., precipitation with ammonium sulfate; E-BC, Emulphogene BC 720; Gp, group.

C. Serotype Outer Membrane Protein Vaccines

Over the past ten years, a number of different group B vaccines based on OMPs have been prepared and tested in animals and in human beings for safety and immunogenicity. Representative vaccines are listed in Table 1. A variety of different procedures for purifying and solubilizing the OMPs were used, but the basic objective was similar in each case: isolation of the major OMPs and removal of the endotoxic LPS by methods that preserve the immunogenicity of the OMPs. The methods varied principally in the detergent used to extract and isolate the OMPs, the method for separating the LPS, and the method of maintaining OMP solubility. Typically, the OMP preparations were enriched for the major OMPs, but they contained a number of minor OMPs and up to 10% to 20% LPS as well.

Most of the vaccines studied, to date, have been noncovalent complexes of OMPs and CP. Our initial studies [47] involved complexes with group B CP because it was anticipated that the antibodies induced by the B CP would be protective.

Frasch and associates [83–86] isolated and tested the OMPs alone as a vaccine. Depending on which method was used, the OMP vaccines were either insoluble (particulate) or relatively soluble. Although these products produced protective antibodies in animals, the response in human volunteers to the less-soluble vaccines was relatively poor. Comparisons of OMP vaccines, with and without added CP, revealed that addition of the CP improved solubility and immunogenicity.

When it became evident that unmodified group B CP probably would not induce protective antibodies and could, in principle, have an adverse effect, CPs from other serogroups were used in place of the group B CP and were equally effective. As a step toward a vaccine applicable to use in military recruits, we prepared a OMP vaccine consisting of OMPs from two prevalent group B serotypes, 2b:P1.2 and 15:P1.16, complexed to a tetravalent A, C, Y, W135 mixture of CPs. This vaccine was tested for safety and immunogenicity using a single dose in adult volunteers in two different studies [45,89,90]. The vaccine induced a normal response to the CPs, and it induced a fourfold or greater bactericidal antibody response to the OMPs of both strains in about two-thirds of the volunteers.

Frasch and co-workers have shown that adsorption of OMP vaccines to aluminum-based adjuvants significantly enhance OMP immunogenicity in mice and in human volunteers [88,91]. Adsorption at pH 7 at a 1:100 w/w ratio of antigen/adjuvant gave the best results in mice, both aluminum hydroxide and aluminum phosphate were effective as adjuvants.

One problem associated with the production and use of noncovalent CP-OMP complexes as vaccines is the difficulty in reproducing the degree of complexing. The terminal lipid moiety on the CP is attached by ester linkages that are relatively unstable [46]. As a result, the percentage of the CP chains that have the terminal lipid moiety present varies from one preparation to the next. During preparation of the OMP-CP complexes, further degradation may occur because of the action of phospholipases [92] that may be present in the OMP preparations. Since the degree of CP binding determines the solubility of the OMPs and, thereby, affects the antigenicity, it is important to be able to standardize the interaction. Moreno et al. [93] have approached this problem by using gel filtration to separate OMP-bound CP from free CP and, thus, obtain a more-reproducible, highly complexed product.

Poolman and co-workers [94] investigated alternative means of maintaining OMP solubility and found that a relatively low concentration of the zwitterionic detergent

Zwittergent 3,14 was as effective as group C CP in enhancing the solubility and immunogenicity of meningococcal OMPs. Animal studies of toxicity and reactogenicity of Zwittergent-solubilized vaccines showed them to be well tolerated [95].

Teerlink et al. [96] found that addition of detergent and adsorption to aluminum hydroxide produced additive adjuvant effects on the immunogenicity of gonococcal OMP in mice.

Because there is evidence that LPS can induce bactericidal antibodies [97,98], we investigated the use of alkaline detoxified LPS (dLPS) of immunotypes L3 and L8 to solubilize the OMPs. The alkaline treatment resulted in removal of all ester-linked fatty acids from the lipid A, without disturbing the amide-linked fatty acids. The dLPS was 1000- to 10,000-fold less toxic than untreated LPS and was able to bind hydrophobically to OMPs through the remaining fatty acids [98]. The resulting complexes were soluble and immunogenic in mice and rabbits. Several advantages of using the dLPS for solubilization of the OMPs include the incorporation of an additional antigen capable of inducing bactericidal antibodies with relatively broad specificity; greater stability and reproducibility of the noncovalent complexes; and suitability for use in a multidose vaccination schedule.

A second problem in the production of OMP vaccines is the variable amount of residual LPS present. Different OMP vaccines have contained from less than 1% to more than 20% LPS relative to OMP. It is apparent from pyrogen tests and safety studies with these vaccines that LPS that is tightly bound to the OMPs does not express its full biological activity [48]. The actual role that residual LPS plays in the reactogenicity, immunogenicity, and protective effect of OMP vaccines is now unclear, but possible effects include the induction of anti-LPS antibodies, expression of adjuvant activity, stabilization of the conformation of OMPs to which it is bound, and increased reactogenicity. Studies with more highly purified OMPs will be required to resolve this question.

The results from clinical studies with different OMP vaccines have led to the following general conclusions about the human immune response to meningococcal OMPs:

1. Safety studies indicate that OMP vaccines are safe, in spite of relatively high levels of LPS in some of them [44,86,89]. Mild systemic effects such as fever, headache, or nausea may be experienced by up to 10% of volunteers. Local reactions consisting of erythema, with or without induration and soreness, are experienced by most adult volunteers [86], but the frequency of local reactions is less in children [85].
2. In various studies, 60% to 90% of volunteers responded with a fourfold or greater increase in bactericidal antibodies to strains of the homologous serotype and subtype [45,86]. Somewhat higher percentages are obtained by enzyme-linked immunosorbent assay (ELISA), but the correlation between IgG ELISA and bactericidal assays is only fair, and it is poor for other antibody classes [89].
3. The specificity of the human antibody response to the OMPs is heterogeneous. Western blots show responses to a number of different OMPs including, but not limited to, the major OMPs [71,99]. Different individuals respond to different OMPs. Overall, most of the bactericidal antibodies are serotype- or subtype-specific, but some cross-reactive antibodies are also induced [45].
4. Antibody levels decrease after vaccination, but remain above prevaccination levels for at least one year (unpublished data). A booster dose given at about six

weeks usually results in an increase in antibodies to a level slightly above the two-week value [47,86].

5. Adsorption to aluminum adjuvants generally results in a modest enhancement of the antibody response [88,91], particularly with the first dose.

A small efficacy trial involving a serotype 2a OMP group B CP vaccine prepared by Frasch et al. [44] was carried out in Capetown, South Africa in 1981. A total of 4440 children, ages four months to five years, were vaccinated with either the experimental vaccine (2220 children) or a control A-C vaccine (2220 children) in a double-blind study. Unfortunately, the number of cases involved was not large enough to obtain statistically significant results.

More recently, efficacy trials were undertaken by Campa and associates in Cuba with serotype 4:P1.15 vaccine, prepared in Cuba generally according to the methods of Frasch [27], and by Boslego and associates (unpublished) in Chile with a serotype 15:P1.3 vaccine prepared by Connaught Laboratories, Inc., generally according to the methods of Zollinger et al. [98]. Both vaccines were noncovalent complexes with group C CP. The results of these trials are not yet available.

New approaches to the development of an OMP vaccine based on the class 1 or subtype OMP have begun with the cloning of the structural gene for the serotype 16 class 1 protein of strain MC50 [100]. The cloning of the gene for the class 1 OMP will facilitate determination of the primary structure of this OMP and, possibly, its purification in an antigenically active form. A covalent conjugate of the class 1 protein and LPS-derived oligosaccharides has been suggested as a possible vaccine [81]. In addition, the subtype epitope on this OMP is quite stable [73]; therefore, it is possible that conserved amino acid sequences can be identified that could be synthesized and form the basis of a synthetic peptide vaccine.

D. Lipopolysaccharide Vaccines

The LPS of *N. meningitidis* is a particularly potent endotoxin [101] and, as a result, the research on its use in group B vaccines has lagged somewhat behind the work on OMPs. All LPSs of *N. meningitidis* are of the rough variety and are often referred to as *lipooligosaccharides* [102,103]. A given strain may possess multiple LPS immunotype determinants that are manifest as distinct bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [104-106]. Common determinates also have been identified and may be present on more than one band [103]. The chemical structures of the oligosaccharides corresponding to some of the immunotypes have been determined, primarily by Jennings et al. [107]. These studies have revealed a basic oligosaccharide structure that is common to most of the immunotypes and consists of an inner core and a lacto-*N*-neotetraose branch. The different immunotype structures are formed by different substitutions on the heptose residues of the inner core that involve only one or two sugar residues and an ethanolamine phosphate group.

Although the lacto-*N*-neotetraose branch is large, accessible, and in a terminal position, it is generally immunorecessive. This can be explained by its extensive structural homology to host tissue antigens [108]. Mandrell and colleagues demonstrated immunochemical similarity between structures on neisserial LPS and paragloboside, a precursor to human blood group antigens, by means of several mouse monoclonal antibodies against highly conserved epitopes on *N. gonorrhoeae* LPS. These antibodies,

which were able to aggluinate human red blood cells at 4°C, bound to some, but not all, *N. meningitidis* LPS types [108].

Several lines of evidence support the use of LPS determinants in a group B vaccine. The LPSs induce antibodies after natural infections [109,110], and some of these have bactericidal activity [97]. Some monoclonal antibodies to LPS immunotype-specific determinants are bactericidal and are passively protective in an infant rat model [81]. Several of the LPS immunotypes, particularly type L3,7, are quite broadly shared among group B case strains. In a survey of 83 case strains from the U.S. Army and from Norway we found the L3,7 or L3,7,8 immunotype was present on over 85% of the isolates (unpublished data). A vaccine containing a mixture of only three or four immunotypes could conceivably protect against most group B disease. The antigenic determinants of the LPS tend to be very stable structures and remain antigenic when detoxified [98] or converted to oligosaccharides by mild acid hydrolysis [111].

There are also several problems associated with the use of the LPS in a vaccine. The most obvious is its toxicity, but the LPS can be readily detoxified by mild acid hydrolysis, which cleaves off the lipid A, or by mild alkaline hydrolysis, which removes ester-linked fatty acids from the lipid A. A second problem is the method of presentation of the oligosaccharide or detoxified LPS. Presented alone, these molecules have poor immunogenicity and must generally be linked to a carrier molecule to be immunogenic. A third problem is the instability of expression of LPS determinants. The meningococcus, like the gonococcus, appears to have the capability of switching on or switching off expression of certain of the immunotype determinants at a relatively high frequency [112; unpublished data]. Finally, there is the problem of choosing the most appropriate epitopes for inclusion in a vaccine.

Jennings and co-workers [111] have prepared dephosphorylated oligosaccharides from the LPS of five different immunotypes and covalently conjugated them to tetanus toxoid as their 2-(4-isothiocyanatophenyl)-ethylamine derivatives for presentation as vaccines. The conjugates, when injected into rabbits, induced bactericidal antibodies that were mostly immunotype-specific, but were sometimes cross-reactive. These studies demonstrated the feasibility of this approach, but they need to be extended to human studies and to the examination of alternative protein carriers, such as group B OMPs.

We have extended the concept of using noncovalent complexes to include vaccines consisting of alkaline-detoxified LPS hydrophobically complexed to the major OMPs [98]. An advantage of this approach is that the LPS is minimally altered and is bound to chemically unmodified OMPs in essentially the same way as it is on viable cells. Two vaccines, each containing OMPs of serotypes 2b:P1.2 and 15:P1.16 complexed to detoxified LPS of immunotype L3 or L8, were tested for immunogenicity in rabbits. Two subcutaneous injections of 100 μg total protein and 100 μg dLPS in saline were given six weeks apart. The vaccines induced geometric mean 45-fold increases in anti-LPS antibodies and 80-fold increases in anti-OMP antibodies. Bactericidal antibody titers against the homologous strains rose 64-fold (unpublished data). Thus noncovalent complexes containing dLPS and OMPs may also have potential for use as a group B vaccine.

E. Cross-reactive Outer Membrane Protein Vaccines

1. H.8 Antigen

In 1984, Cannon and co-workers [75] reported the isolation of a monoclonal antibody H.8 which bound to a protein determinant present in the outer membrane of all pathogenic

Neisseria and most *N. lactamica*. The H.8 antigen, as it has come to be called, appears to be a lipoprotein with a relative molecular mass in the range of 20,000 and 30,000 depending on the strain. It was purified from the meningococcus and the gonococcus [113-115] and found to consist primarily of three amino acids (alanine, proline, and glutamic acid) that accounted for over 80% of the total amino acids [119]. No aromatic amino acids were present, which was consistent with its lack of absorbance at 280 nm and failure to stain with Coomassie Blue. Amino acid sequencing of the protein was unsuccessful, apparently because of a blocked NH_2-terminus. Fatty acid analysis of the purified antigen revealed the presence of one or more, as yet unidentified, lipid components [114,115].

The structural gene for the H.8 antigen has been cloned from the gonococcus [116] and, subsequently, also from the meningococcus [117]. Gotschlich et al. [118] reported the presence of three distinct genes in the gonococcal genome and two different gene products that were expressed and reacted with the H.8 antibody. Two genes were subsequently also reported for the meningococcus [117]. The amino acid sequence of one of the gene products was determined from the 5′ DNA sequence of the structural gene and found to contain a signal sequence characteristic of lipoproteins followed by a 39-amino acid region rich in alanine and proline and containing the epitope that reacts with the H.8 antibody. The amino acid sequence, as determined from the DNA sequence, did not agree well with the amino acid composition of the purified antigen as reported by Bhattacharjee et al. [115], except for the NH_2-terminal 39-amino acid region containing the epitope recognized by the H.8 antibody. The sequences that have been reported [117,119] may therefore not correspond to the actual H.8 antigen that was purified, but rather to the gene product of the second gene.

The potential of the H.8 antigen as a possible vaccine candidate is being explored. Hitchcock et al. [120] used gold sphere immunologic probes and found the H.8 antigen to be surface exposed in a variable manner in that some cells bound the antibody-coated spheres and some did not. Monoclonal antibodies specific for H.8 have been reported to lack bactericidal activity and failed to provide passive protection in a mouse model [121]. On the other hand, good antibody responses to H.8 after natural infections are demonstrable in human sera by Western blotting, and by ELISA, using purified H.8 antigen [122,123]. Although some of the data suggest that H.8 may not be effective as a vaccine, it has other properties, such as surface exposure, stable linear epitope, consistent expression in all pathogenic *Neisseria*, and good antigenicity, that make it an attractive vaccine candidate.

2. *Other Outer Membrane Proteins*

Several other surface-exposed or extracellular proteins have been suggested as candidates for a group B or general meningococcal vaccine, either because of a high degree of structural conservation or because they are believed to play an important role in pathogenesis, or both. The expression of certain OMPs has been shown to be regulated by the availability of iron. Studies with iron-regulated proteins, with relative molecular masses of 37 kDa, 70 kDa, and 94 kDa [124,125], have demonstrated that these proteins are surface exposed [126], expressed in vivo, and elicit antibodies during the course of natural infections [125]. The proteins are quite highly conserved, or at least contain epitopes that are highly conserved, among pathogenic *Neisseria* as well as some nonpathogenic *Neisseria* [124]. The 37-kDa major iron-regulated protein has been purified from

both the gonococcus and the meningococcus, and the amino acid composition and NH_2-terminal amino acid sequences compared [127]. It is not known whether antibodies to the iron-regulated proteins are bactericidal or protective.

The IgA proteases and pili are thought to play an important role in pathogenesis at the level of the mucosal membranes. Antibodies that neutralize the normal functions of these proteins may be effective in preventing infection. The structural genes for the IgA1 protease and pilin of the gonococcus have been cloned and sequenced. These studies are discussed in Chapter 34 on gonococcal vaccines. Significant structural homology and serological cross-reactivity exists between the gonococcal and meningococcal forms of these proteins [128,129], as well as the OMP macromolecular complex [130] and the class 4 OMP (gonococcal protein III) [131]. This suggests that a vaccine based on one or more of these cross-reactive proteins may have applicability to both species.

III. IMPROVED VACCINES FOR GROUPS A AND C

Prospects for improving the immunogenicity of the purified meningococcal CP vaccines in children younger than two years of age seem good in view of the success achieved with conjugates of *H. influenzae* type b CP and carrier proteins [132,133]. The T-cell-independent CP antigen is converted to a T-cell-dependent antigen by conjugation to an appropriate carrier protein that supplies the needed T-cell epitopes.

Although the principle has been established as valid by the work with *H. influenzae*, there are several important issues that need to be resolved for optimal application of the principle to meningococcal CPs. The choice of the CP; the method of conjugation; the optimal CP/protein ratio; and the methods of standardization, storage, and presentation of the vaccine are among the questions to be resolved. Because of the lack of a group B vaccine, it is attractive to consider conjugates of CPs (e.g., groups A and C) with group B OMPs. A conjugate involving group B serotype 2a OMP has been effective for the *H. influenzae* type b vaccine [133], but minimal attention has been given to examining the immunogenicity of the protein part of the conjugate. If group B OMPs are used to prepare conjugates, it will be particularly important to do the conjugation in a way such as to preserve the full antigenicity of proteins as well as the CPs. Group C CP-OMP conjugates have been prepared [134], but much more work needs to be done with equal emphasis on both parts of the conjugate.

Most of the conjugation work with meningococcal CPs has been done using tetanus toxoid as the carrier protein. Tetanus and diphtheria toxoids are attractive from the viewpoint of solubility, their extensive use as vaccines, and the likelihood of fewer problems with standardization. On the other hand, the administration of several conjugate vaccines containing these toxoids could result in carrier-induced epitopic suppression [135].

Beuvery et al. [136,137] studied several different methods of conjugation with tetanus toxoid as carrier protein for group C and group A CPs. Coupling with *N*-ethyl-N'-(3-dimethylaminopropyl)carbodiimide (EDC) and the use of a 6-amino-*n*-hexanoic acid spacer produced a conjugate with greater immunogenicity than when the coupling was done with cyanogen bromide. Jennings and Lugowski [52] used a third method which involved the introduction of reactive aldehyde groups on the terminal residues of the CPs of groups, A. B, and C by treatment with metaperiodate, followed by conjugation to tetanus toxoid by reductive amination with sodium cyanoborohydride. The group A and group C conjugates produced good group A- and group C-specific antibody responses

in rabbits, but the B conjugate induced mainly antibodies to the linkage point that did not react with the native CP. Beuvery and associates [138,139] compared the EDC method with the reductive amination method and found both conjugates to have good immunogenicity. The dose-response and the effect of adsorption to aluminum phosphate, however, were dependent on the method of conjugation.

The feasibility of coupling several different oligosaccharides to a single carrier protein was demonstrated by Porro et al. [140] by coupling oligosaccharides derived from *Streptococcus pneumoniae* type 6A and meningococcal group C polysaccharides to the CRM197 mutant protein related to diphtheria toxin. This approach may be useful in preparing multivalent conjugate vaccines.

IV. FUTURE APPROACHES

The application of modern technology has opened up some interesting and promising possibilities for correcting the deficiencies in the meningococcal vaccine repertoire. The development of a group B vaccine effective against all or most B strains will require either using a modified group B CP with much enchanced immunogenicity, a multivalent serotype-specific OMP or LPS vaccine, or a conserved OMP or peptide. By means of monoclonal antibodies, several specific antigens have been identified as good vaccine candidates. Structural studies, including identification of conserved, surface-exposed epitopes, will be facilitated by the cloning of the respective genes. The most effective methods for isolating these antigens and presenting them in a safe and antigenically active manner will be a principal focus of further research.

Several meningococcal OMP genes have been cloned, including the genes for the H.8 antigen and the class 1 OMP, and structural studies of these antigens are progressing. The successful cloning of the structural genes for specific subcapsular antigens that have been identified as possible vaccine candidates should enable one to prepare new vaccines that include only those antigens or epitopes that elicit protective antibodies. Because certain antigens or epitopes may induce blocking antibodies, or have other undesirable effects, it may be as important to exclude certain antigens or epitopes from a vaccine as it is to include others. In addition, gene cloning opens up new approaches to vaccine presentation, such as use of live, nonpathogenic vectors for vaccine delivery.

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